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Identification of α_1-adrenoceptor subtypes in the rat vas deferens: binding and functional studies

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1 The α_1-adrenoceptor subtypes of the prostatic and epididymal portion of rat vas deferens were characterized in binding and functional experiments.

2 In saturation experiments, [^{3}H]-prazosin bound to two distinct affinity sites in the epididymal portion of rat vas deferens ($pK_D = 10.1 \pm 0.13$ and 9.01 ± 0.15, $B_{max} = 507$ and 1231 fmol mg^{-1} protein, respectively). In the prostatic portion [^{3}H]-prazosin bound to a single affinity site ($pK_D = 9.82 \pm 0.04$, $B_{max} = 924$ fmol mg^{-1} protein).

3 In the displacement experiments, unlabelled prazosin displaced biphasically the binding of 200 pM [^{3}H]-prazosin to the epididymal portion; the resulting two pK_I values were consistent with the affinity constants obtained in the saturation experiments. WB4101 (2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane) and benoxathian also discriminated the two affinity sites in the epididymal portion and the population of low affinity sites for the three antagonists was approximately 40%. On the other hand, the prostatic portion predominantly showed a single affinity site for prazosin, WB4101 and benoxathian, although the presence of a small proportion (less than 10%) of the low affinity site could be detected. HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl) benzeneacetonitrile fumarate) displaced the [^{3}H]-prazosin binding monophasically with a low affinity in both halves.

4 Pretreatment with chlorethylclonidine (CEC) at concentrations higher than 1 μM inhibited 700 pM [^{3}H]-prazosin binding to the prostatic portion by approximately 50%. However, the inhibition in the epididymal portion was much less (approximately 21% at 50 μM CEC).

5 In the functional study, the contractile response to noradrenaline was competitively inhibited by prazosin, WB4101, benoxathian and HV723 with similar and low affinities (pK_B value ranging from 8.0 to 9.0) in the epididymal portion of rat vas deferens. In the prostatic portion of rat vas deferens, noradrenaline also produced a contraction, but the maximal amplitude of contraction developed was approximately one-fourth of that in the epididymal portion. Prazosin and WB4101 also inhibited the contractile response of the prostatic portion with the pK_B values similar to those obtained in the epididymal portion. The contractions to noradrenaline in both portions were potently attenuated by 1 μM nifedipine but were not affected by pretreatment with 10 μM CEC.

6 Under conditions where P_{2x}-purinoceptors and prejunctional α_2-adrenoceptors were blocked, electrical transmural stimulation produced a rapidly developing phasic contraction and a subsequent tonic contraction in the epididymal portion of rat vas deferens. The phasic and tonic contractions were inhibited in a concentration-dependent manner by prazosin ($IC_{50} = 25.7$ and 25.9 nM, respectively), WB4101 ($IC_{50} = 7.27$ and 7.58 nM), benoxathian ($IC_{50} = 10.9$ and 8.66 nM) and HV723 ($IC_{50} = 15.9$ and 14.9 nM). Nifedipine selectively attenuated the tonic contraction induced by electrical stimulation, and the residual phasic response was inhibited by the antagonists mentioned above with similar affinities to those in the absence of nifedipine. CEC (10 μM) had little effect on the adrenergic neurogenic contractions.

7 The present results indicate the presence of two distinct α_1-adrenoceptor subtypes in the rat vas deferens, which show respectively high and low affinities for each of prazosin, WB4101 and benoxathian, and presumably correspond to putative α_{1A} and α_{1L} subtypes according to the recent α_1-adrenoceptor subclassifications. The contractions induced by exogenous and endogenous noradrenaline seem to be predominantly mediated through the α_{1L} subtype. The heterogeneous distribution of the low affinity sites (α_{1L} subtype) may well explain differences in functional responsiveness between the two portions of rat vas deferens.

Keywords: α_1-Adrenoceptor subtype; noradrenaline-induced contraction; rat vas deferens; α_1-adrenoceptor subclassification

Introduction

The α_1-adrenoceptors are not homogeneous in all tissues and it has been suggested that their heterogeneity may be related, in part, to the presence of different α-adrenoceptor subtypes (Bülbring & Tomita, 1987; McGrath & Wilson, 1988; Minneman, 1988; Wilson *et al.*, 1991). Recent radioligand binding studies with [^{3}H]-prazosin or [^{125}I]-BE2254 (2-[β-4-hydroxy-3-[^{125}I]-iodophenyl)-ethylaminoethyl]-tetralone) demonstrated two separate populations of α_1-adrenoceptors in the rat brain and the rat vas deferens which were designated α_{1A} (or α_{1a}) and α_{1B} (or α_{1b}), respectively (Morrow & Creese, 1986; Han *et al.*, 1987a). The α_{1A} (or α_{1a}) subtype has high affinity for (2-(2,6-dimethoxy-phenoxyethyl-amino methyl-1,4-benzodioxane (WB4101), benoxathian and phentolamine, while the α_{1B} (α_{1b}) subtype has lower affinity for the competitive antagonists and is potently inactivated by chlorethylclonidine (CEC) (Han *et al.*, 1987b). However, the subtypes cannot be discriminated by prazosin and yohimbine (Hanft & Gross, 1989). In contrast, results of functional studies with blood vessels have suggested another subclassification, where α_1-adrenoceptors can be classified into two (α_{1H}, α_{1L}) or three (α_{1H}, α_{1L} and α_{1N}) subtypes by their different affinities for

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prazosin and α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl benzeneacetonitrile fumarate (HV723) (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990b). We have recently demonstrated that the α_{1A} and α_{1B} subtypes can be identified as a single site with high affinity for prazosin, suggesting that α_{1A} and α_{1B} subtypes may be included in the α_{1H} subtype defined in the α_{1H}, α_{1L}, α_{1N} subclassification (Oshita *et al.*, 1991; Muramatsu *et al.*, 1991). Further, we demonstrated the low selectivity of CEC for α_1-adrenoceptor subtypes and proposed a possible conciliation of the two distinct α_1-adrenoceptor subclassifications as shown in Table 1.

The α_1-adrenoceptors of rat vas deferens have so far been studied according to the α_{1A} and α_{1B} subclassification and the presence of both the subtypes has been suggested (Han *et al.*, 1987a; Hanft & Gross, 1989). However, the occurrence of only α_{1A} but not α_{1B} subtype in the rat vas deferens was recently confirmed by molecular biological approaches (Lomasney *et al.*, 1991b). The contractile response to noradrenaline has been reported to be mediated through the α_{1A} subtype which is closely coupled to voltage-dependent Ca channels (Han *et al.*, 1987a; Minneman, 1988), whereas the adrenergic neurogenic contraction induced by electrical stimulation with a single pulse is completely resistant to nifedipine (Blakeley *et al.*, 1981; McGrath & Wilson, 1988; Spriggs *et al.*, 1991). Further, prostatic and epididymal portions of rat vas deferens differ not only in their neuro-effector transmission (McGrath, 1978; Brown *et al.*, 1983) but also in their postjunctional responses to various α-adrenoceptor agonists (Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982; Badia & Salles, 1989). Such evidence suggests complexity of the adrenergic transmission in rat vas deferens which should be resolved. In the present paper, we characterized the α_1-adrenoceptor subtypes in the epididymal and prostatic portions of rat vas deferens on the basis of the criteria defined in the α_1-adrenoceptor subclassifications mentioned above.

Methods

Binding study

Vasa deferentia were isolated from male Wistar rats (260–450 g) and cut into two (prostatic and epididymal) portions. Each portion was separately homogenized in 100 vol of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a polytron (setting 8, 15 s × 2). The homogenates were filtered through 4 layers of gauze and centrifuged at 80,000 *g* for 20 min at 4°C. The pellets were resuspended in the same volume of assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated for 20 min at 37°C, and centrifuged again as described above. All procedures to prepare membranes were conducted at 4°C except preincubation of the membranes, and ice cold buffers were used. The final pellet was resuspended in assay buffer and used for the binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml in all experiments. Reactions were terminated by rapid filtration using a Brandel cell harvester on to Whatman GF/C filters. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried and the filter-bound radioactivity determined. Non-specific binding was defined as binding in the presence of 1 or 10 μM prazosin. Assays were conducted in duplicate.

Table 1 Putative α_1-adrenoceptor subtypes and relative affinities for representative competitive antagonists

	Relative affinity		
α_1-subtype	*Prazosin*	*WB4101*	*HV723*
α_{1H}—α_{1A}	High	High	Medium or Low
α_{1H}—α_{1B}	High	Low	Low
α_{1L}	Low	Low	Low
α_{1N}	Low	Low	High

Chlorethylclonidine treatment

Membrane preparations were incubated for 30 min at 37°C with 1, 10 and 50 μM CEC and centrifuged at 80,000 *g* for 20 min. The pellets were washed once with assay buffer before the binding experiment.

Binding data were analysed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares was statistically less for a two-site fit of the data than for a one-site, as determined by *F*-test comparison, then the two-site model was accepted. *P* values less than 0.05 were considered significant.

Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Functional experiments

Vas deferens of male Wistar rats (260–350 g) was isolated and cut into two (prostatic and epididymal) portions. Each portion was mounted vertically in an organ bath containing 20 ml Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, $NaHPO_4$ 1.2 and glucose 11.5. The medium was maintained at 37°C, pH 7.4 and was equilibrated with a gas mixture consisting of 95% O_2 and 5% CO_2. A resting tension of 0.5 g was applied and the responses were recorded isometrically through a force-displacement transducer. The preparations were equilibrated for 90 min before starting the experiments.

Concentration-response curves for noradrenaline were obtained by adding the drug directly to the bathing media in a non-cumulative fashion. Desmethylimipramine (0.1 μM), deoxycorticosterone acetate (5 μM) and propranolol (3 μM) were present throughout this series of experiments in order to block neuronal and extraneuronal uptake of noradrenaline and β-adrenoceptors, respectively. α-Adrenoceptor antagonists were present for 30 min or 60 min before and during the contractile-response to noradrenaline. With CEC treatment, the preparations were treated once for 20 min with 10 μM CEC and then washed repeatedly with drug-free solution.

The pK_B value was estimated according to Arunlakshana & Schild (1959). Briefly, the concentration of noradrenaline necessary to give a half-maximal response in the presence of α-adrenoceptor antagonist was divided by the concentration giving a half-maximal response in the control to determine the agonist concentration-ratio (CR). Data were plotted as the −log antagonist concentration (M) vs the log (CR-1) and pA_2 values were calculated from Schild plots. The mean slope and 95% confidence limits (95% CL) were obtained from straight lines drawn by least square linear regression. When the straight line yielded a slope not significantly different from unit, the pA_2 value estimated was represented as pK_B (Arunlakshana & Schild, 1959). In the prostatic portion of rat vas deferens, the pK_B value for α-adrenoceptor antagonist was determined for single concentrations of antagonist (10 or 100 nM) by the concentration-ratio method (Furchgott, 1972).

Electrical transmural stimulation was applied through a pair of platinum-wire electrodes at 10–15 min intervals (Muramatsu *et al.*, 1989). The preparation was placed in parallel between electrodes. The distance between the electrodes was approximately 3 mm. Stimulus parameters were 0.1 ms duration, frequencies of 5 Hz and supramaximal voltage (13 V) for 10 s, unless stated otherwise. In this series of experiments, DG-5128 (10 μM) and propranolol (1 μM) were added to the bath medium to block prejunctional α_2-adreno-

ceptors and β-adrenoceptors, respectively (Muramatsu *et al.*, 1983; 1989). DG-5128 (10 μM) had no effect on the contractile response to noradrenaline in each preparation. α,β-Methylene ATP (10 μM) was also present throughout the experiments in order to block the purinergic component (Brown *et al.*, 1983; Sneddon & Burnstock, 1984). No effect of α,β-methylene ATP on noradrenaline-induced contraction had been established in preliminary experiments.

Statistical analyses

Experimental values are given as a mean ± s.e.mean. Results were analyzed by Student's *t* test and a probability of less than 0.05 was considered significant.

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 76.6 Ci mmol^{-1}, NEN, Boston, U.S.A.), prazosin hydrochloride (Taito-Pfizer, Tokyo, Japan), phentolamine mesylate (Ciba, Basal, Switzerland), WB4101 hydrochloride (2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride), benoxathian hydrochloride, chlorethylclonidine dihydrochloride (CEC) (Funakoshi, Tokyo, Japan) and HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxy-phenoxy)ethyl)-amino)-propyl)benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Fukui, Japan), nifedipine, desmethylimipramine hydrochloride (Sigma, St. Louis, U.S.A.), (−)-noradrenaline bitartrate, deoxycorticosterone acetate, (±)-propranolol hydrochloride (Nacalai, Kyoto, Japan), tetrodotoxin (Sankyo, Tokyo, Japan) and DG-5128 (2-(2-(4,5-dihydro-1H-imidazol-2-yl)-1-phenylethyl) pyridine dihydrochloride sesquihydrate, Daiichi Seiyaku, Tokyo, Japan).

Results

Saturation experiments with [^{3}H]-prazosin

[^{3}H]-prazosin at concentrations ranging from 20–3000 pM was used to label α_1-adrenoceptors of rat vas deferens. The specific binding was approximately 90% of the total binding at 200 pM [^{3}H]-prazosin and showed a saturable tendency at the concentrations of 2000–3000 pM. However, Scatchard plots of the binding data in the epididymal portion were curvilinear, suggesting more than a single class of binding site (Figure 1a). LIGAND analysis fitted the data to a two site model. The pK_D value of high and low affinity sites were 10.1 ± 0.13 and 9.01 ± 0.15, and the B_{max} values for both sites were 507 ± 79 and 1231 ± 563 fmol mg^{-1} protein, respectively ($n = 4$).

On the other hand, Scatchard plots of the data obtained from the prostatic portion were apparently linear, resulting in a better fitting to a one-site model in computerized analysis (Figure 1b). The pK_D value estimated (9.82 ± 0.04, $n = 5$) was close to the value for the high affinity site in the epididymal portion, whereas the B_{max} value (924 ± 175, $n = 5$) was slightly but not significantly greater than that in the epididymal portion. Close inspection of Figure 1b also revealed that the binding of high concentrations of [^{3}H]-prazosin deviates slightly from a straight line, suggesting the possible existence of low affinity sites in a minor proportion.

Effects of competitive antagonists on [^{3}H]-prazosin binding

The pharmacological profile of high and low affinity sites for [^{3}H]-prazosin was further examined in displacement experiments.

Epididymal portion When 200 pM [^{3}H]-prazosin was used, unlabelled prazosin, WB4101 and benoxathian showed shallow displacement curves. However, HV723 displaced the binding in a monophasic manner (Figure 2a). Computerized analysis revealed that prazosin, WB4101 and benoxathian bound to two distinct sites. The high and low pK_I values for prazosin were respectively the same as the pK_D values obtained in the saturation experiments with [^{3}H]-prazosin. The pK_I values at high and low affinity sites for WB4101 or benoxathian were also not significantly different from the values at the corresponding sites for prazosin. The proportion of the low affinity sites for each antagonist was approximately 40% of the total binding sites (Table 2).

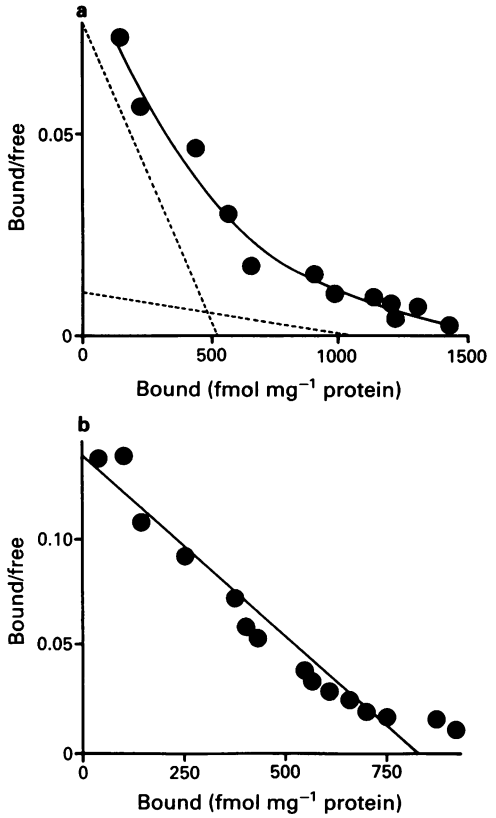

Figure 1 Scatchard plots for specific [^{3}H]-prazosin binding to rat vas deferens membranes in saturation experiments. [^{3}H]-prazosin 20–3000 pM. The figures are obtained from a single experiment where each point is the mean of duplicate determinations: (a) epididymal and (b) prostatic portions of rat vas deferens.

Prostatic portion In three out of four experiments, unlabelled prazosin displaced the binding of 200 pM [^{3}H]-prazosin in a monophasic manner and the pK_I value obtained was consistent with high pK_I value in the epididymal portion (Figure 2b and Table 2). In a remaining experiment, prazosin detected two distinct sites although the proportion of low affinity site was small (less than 10%). WB4101 also produced similar results to those for prazosin. On the other hand, benoxathian and HV723 displaced the binding of [^{3}H]-prazosin in a monophasic manner (Table 2).

Effects of pretreatment with chlorethylclonidine on [^{3}H]-prazosin binding

Since at least two distinct affinity sites for prazosin were detected in the rat vas deferens membranes, we examined the effects of pretreatment with various concentrations of CEC. In this series of experiments, 700 pM [^{3}H]-prazosin was used to label a greater number of binding sites. Therefore, the proportion of the prazosin-low affinity sites in the membranes of epididymal portion increased to approximately 80%, whereas the sites in the prostatic portion were not

clearly detected ($n = 3$ in each portion). Pretreatment of the epididymal portion with CEC at concentrations of 1 and 10 μM did not reduce the number of total specific binding sites, but pretreatment with 50 μM CEC decreased the specific binding by 21 ± 6% ($n = 3$) (Figure 3). On the other hand, [^{3}H]-prazosin binding to the membranes of prostatic portion was significantly reduced by CEC at concentrations higher than 1 μM, but a complete inhibition was not produced even at 50 μM CEC.

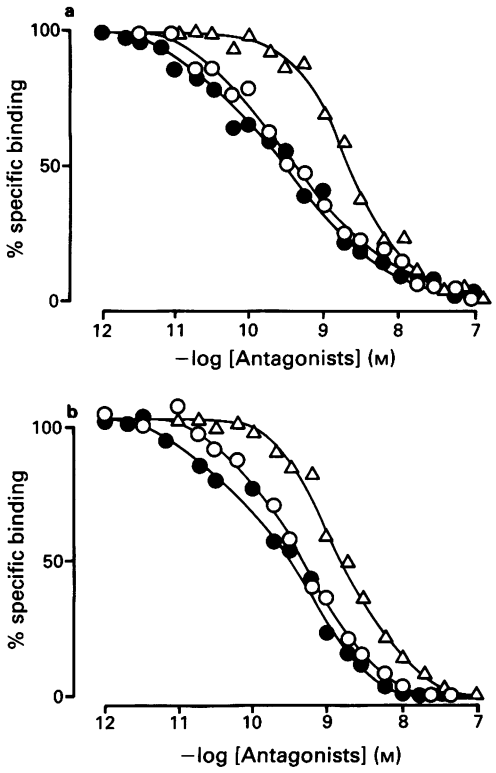

Figure 2 Displacement of [^{3}H]-prazosin binding from (a) epididymal and (b) prostatic portions of rat vas deferens membranes by prazosin (●), WB4101 (○) and HV723 (△). [^{3}H]-prazosin (200 pM) was incubated with various concentrations of unlabelled drugs under the assay conditions described in Methods. The figure represents a single experiment for each drug, where each point is the mean of duplicate determinations.

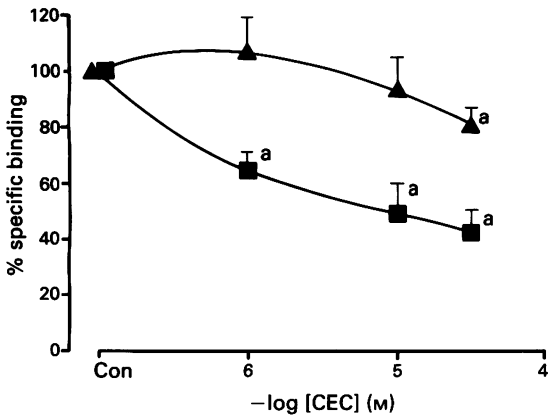

Figure 3 Effects of pretreatment with chlorethylclonidine (CEC) on the specific binding of [^{3}H]-prazosin (700 pM) to epididymal (▲) and prostatic (■) portions of rat vas deferens. The ordinate scale represents relative values of specific binding in CEC-treated membranes to that in CEC-untreated membranes (Con). Each value is the mean of 3 experiments with s.e.mean shown by vertical lines. [a]Significantly different from the value in CEC-untreated membranes ($P < 0.05$).

Effects of nifedipine and chlorethylclonidine on the contractile responses to noradrenaline

Noradrenaline at concentrations in excess of 100 nM produced concentration-dependent contractions both in the epididymal and prostatic portions of rat vas deferens (Figure 4). The pD_2 value (6.01 ± 0.10) in the epididymal portion was significantly higher than that (5.14 ± 0.17) in the prostatic portion ($P < 0.05$, $n = 5$ in each portion). The maximum amplitude of contractions in the epididymal portion was also approximately four times greater than that in the prostatic portion. Pretreatment with 10 μM CEC failed to affect the contractions induced by noradrenaline in both portions. However, the contractions were potently attenuated by 1 μM nifedipine (Figures 4 and 5a).

Effects of prazosin, WB4101, benoxathian and HV723 on noradrenaline-induced contractions in the epididymal and prostatic portions of rat vas deferens

The contractile-responses to noradrenaline in the epididymal portion were attenuated by prazosin, HV723, WB4101 and benoxathian. The slopes of Schild plots were close to unity for all the antagonists tested, indicating that the four anta-

Table 2 Inhibition of 200 pM [^{3}H]-prazosin binding to α_1-adrenoceptor of rat vas deferens

Portion	*Antagonist*	n	*Slope factor*	$pK_{I\ high}$	$pK_{I\ low}$	*% low*
Epididymal	Prazosin	4	0.50 ± 0.04[a]	10.50 ± 0.19	8.47 ± 0.42	37.9
	WB4101	4	0.75 ± 0.03[a]	10.18 ± 0.47	8.96 ± 0.35	42.1
	Benoxathian	4	0.78 ± 0.04[a]	10.10 ± 0.11	8.80 ± 0.12	48.6
	HV723	3	0.98 ± 0.02	8.90 ± 0.71	–	–
Prostatic	Prazosin	1	0.70	9.98	8.29	7.6
		3	0.87 ± 0.09	10.28 ± 0.31	–	–
	WB4101	1	0.78	9.71	8.14	7.7
		3	0.90 ± 0.03	9.47 ± 0.07	–	–
	Benoxathian	4	0.92 ± 0.07	9.58 ± 0.29	–	–
	HV723	4	0.95 ± 0.02	8.89 ± 0.04	–	–

Data shown are mean ± s.e.mean., n = number of experiments
Displacement experiments were done with 200 pM [^{3}H]-prazosin.
$pK_{I\ high}$ and $pK_{I\ low}$: negative log of the equilibrium dissociation constants (−log M) at prazosin-high and low affinity sites for antagonists tested.
% low: population binding at the low affinity site compared to the total specific binding sites.
[a]Significantly different from unity ($P < 0.05$).

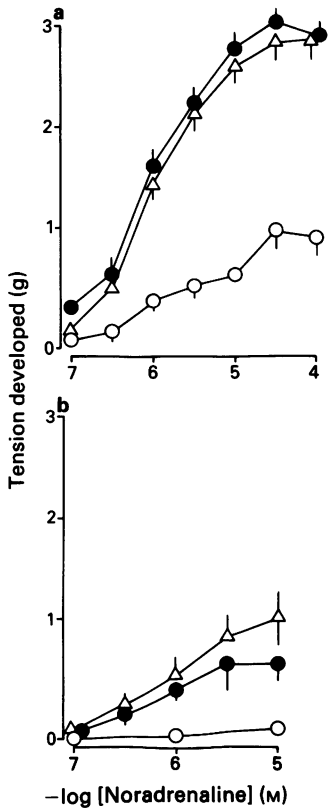

Figure 4 Effects of 10 μM chlorethylclonidine (CEC) and 1 μM nifedipine on the concentration-response curves for noradrenaline in the (a) epididymal and (b) prostatic portions of rat vas deferens. Control response (●); in the responses after pretreatment with CEC (△); or in the presence of nifedipine (○). Each point is the mean of data from 4 to 6 experiments and vertical line shows s.e.mean.

gonists competitively inhibited the contractile responses to noradrenaline. The estimated pK_B values were less than 9.0 for the antagonists tested (Table 3). There was no significant difference in the pK_B values for prazosin or WB4101 between the antagonist equilibration times of 30 and 60 min (Table 3). In the prostatic portion of rat vas deferens also, prazosin and WB4101 inhibited the contractile responses induced by noradrenaline and the pK_B values were similar to the values obtained in epididymal portion of rat vas deferens (Table 3).

Effects of various treatments on adrenergic nerve-mediated contractions in the epididymal portion of rat vas deferens

In the presence of propranolol (1 μM), DG-5128 (10 μM) and α,β-methylene ATP (10 μM), electrical transmural stimulation produced a contraction that consisted of a rapidly developing phasic component and a tonic component lasting during the stimulation (Figure 5b). These responses were completely inhibited by tetrodotoxin (0.5 μM) ($n = 5$). Nifedipine (1 μM) markedly attenuated the tonic component without affecting the phasic response (Figure 5b). Pretreatment with CEC (10 μM) for 20 min slightly attenuated the phasic contractions (15 ± 6% inhibition, $n = 7$) without affecting the tonic responses (Figure 5c). Figure 6 shows the relationship between the stimulus frequency and the contractile amplitude of each phase in the absence or presence of 1 μM nifedipine. The contractile amplitudes were dependent on the stimulus frequencies, resulting in a submaximum value at 5 Hz in each of phasic and tonic responses. Therefore, we examined the effects of α-adrenoceptor antagonists on the neurogenic responses at 5 Hz.

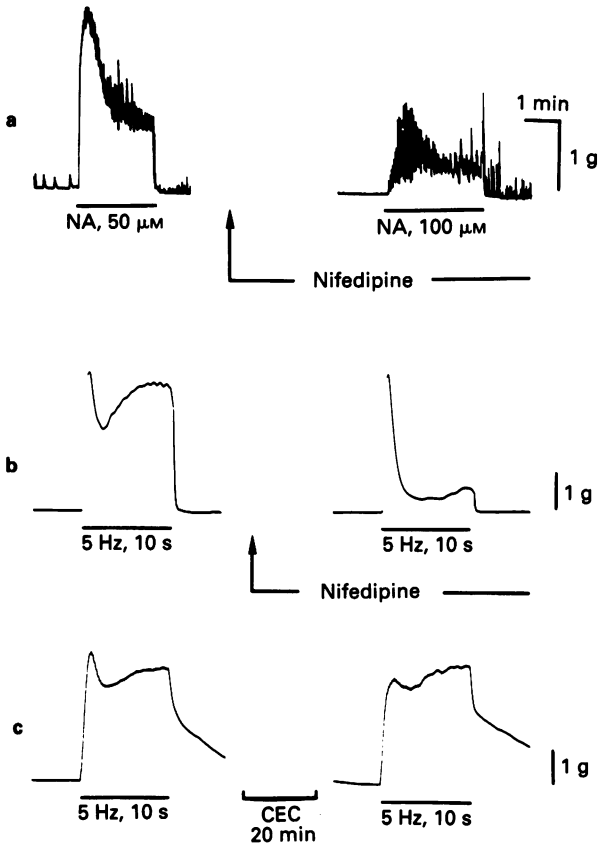

Figure 5 Effects of nifedipine or chlorethylclonidine (CEC)-pretreatment on the adrenergic contractions induced by (a) noradrenaline (100 μM) and (b and c) electrical stimulation (5 Hz, 10 s) in the epididymal portions of rat vas deferens. Left: control, right: response in the presence of 1 μM nifedipine or after pretratment with 10 μM chlorethylclonidine (CEC). In (b) and (c), 1 μM propranolol, 10 μM DG5128 and 10 μM α,β-methylene ATP were present throughout the experiments.

Table 3 α_1-Adrenoceptor affinities for prazosin, HV723, WB4101 and benoxathian in the epididymal and prostatic portions of rat vas deferens

	Epididymal portion		*Prostatic portion*
Antagonist	pK_B	*Slope* (95% CL)	pK_B
Prazosin	8.32 ± 0.05	1.066 (0.999–1.133)	8.37 ± 0.24[b]
	8.39 ± 0.10[a]	1.070 (0.914–1.224)	
HV723	8.22 ± 0.05	1.068 (0.993–1.141)	–[c]
WB4101	8.52 ± 0.08	1.032 (0.932–1.132)	8.75 ± 0.16[b]
	8.70 ± 0.06[a]	1.078 (0.955–1.162)	
Benoxathian	8.41 ± 0.07	1.052 (0.945–1.157)	–[c]

[a]The values were estimated from 60 min equilibration experiments; other values were obtained from 30 min equilibration experiments.
[b]The pK_B values were estimated from the inhibitory effects of 10 and 100 nM prazosin or WB4101.
[c]Not determined.

Prazosin, HV723, WB4101 and benoxathian inhibited concentration-dependently and eventually abolished the contractile responses to electrical transmural stimulation. Figure 7 shows the concentration-inhibition curves for the four antagonists, where the phasic and tonic components in the absence or presence of 1 μM nifedipine were measured separately. The inhibition by WB4101 and benoxathian was slightly more potent than that by prazosin or HV723, but the ratios between the IC_{50} values were in a range less than 4 times (Table 4). Nifedipine (1 μM) did not affect the inhibitory potencies of the antagonists.

Discussion

The present study clearly demonstrates that prazosin binds to two distinct populations of binding sites in the rat vas deferens. However, the density of two sites varied between the epididymal and prostatic portions. The prazosin-high affinity sites were present in almost equal density in both halves, whereas prazosin-low affinity sites predominantly occurred in the epididymal portion and the density was approximately twice that of high affinity sites in the epididymal portion. The proportion of low affinity sites in the prostatic portion was markedly low (less than 10% of total binding sites), so that the low sites could not be consistently detected. This may reflect a limitation of computer analysis (Molinoff *et al.*, 1981; De Lean *et al.*, 1982).

The finding of two distinct binding sites for prazosin in the rat vas deferens is new, only a single affinity site having been demonstrated for prazosin in previous studies (Hanft & Gross, 1989; Salles & Badia, 1991). This discrepancy may be in part associated with the radioligand concentrations used, as the low affinity sites may be overlooked at low concentrations of radioligand. Use of the membrane fraction prepared from whole vas deferens may also mask the prazosin-low affinity sites, because these sites mainly occur in the epididymal portion which corresponds to approximately 35% of the whole vas deferens in protein content, resulting in an apparent reduction of the relative proportion of low affinity sites in total membrane fractions. However, Salles & Badia (1991) failed to detect the low affinity sites even though they used high concentrations of [^{3}H]-prazosin (up to 4 nM) and membranes prepared separately from the epididymal and prostatic portions. One of the reasons for this conflicting result may be a difference in the methods used to determine specific binding, as 10 μM phentolamine may be insufficient to inhibit completely the specific binding of high concentra-

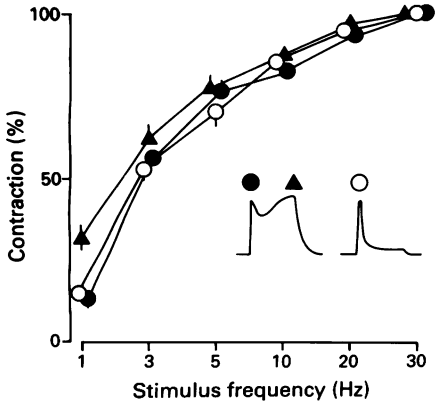

Figure 6 Relation between the contractile amplitudes and stimulus frequencies in the epididymal portion of rat vas deferens. The phasic and tonic contractions induced by stimulation with various frequencies for 10 s were measured as shown in the inset. Closed and opened symbols show the responses in the absence and presence of 1 μM nifedipine, respectively. Other experimental conditions were the same as those in Figure 5b.

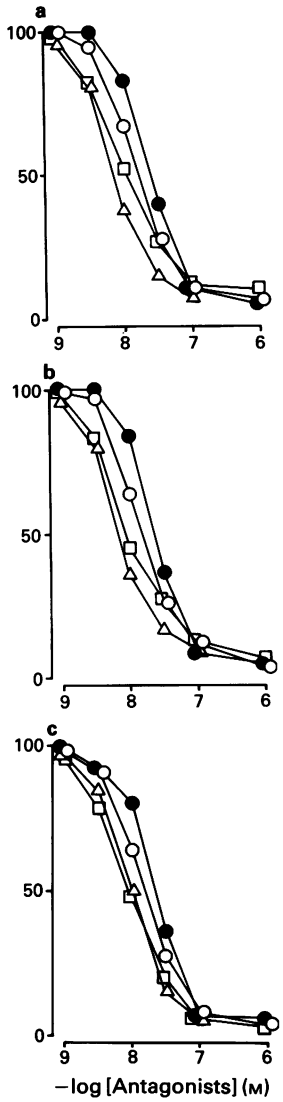

Figure 7 Concentration-response curves for prazosin (●), WB4101 (△), benoxathian (□) and HV723 (○) in inhibiting the adrenergic neurogenic contractions in the epididymal portion of rat vas deferens. Adrenergic neurogenic contraction was elicited by the application of electrical transmural stimulation (5 Hz, 10 s). The other experimental conditions are the same as those in Figure 5b. The contractile amplitudes before addition of prazosin, WB4101, benoxathian and HV723 were taken as 100%. Each value is the mean of 4–5 experiments. (a) and (b) Phasic and tonic contractions in the absence of nifedipine (see the inset of Figure 6); (c) phasic contraction in the presence of 1 μM nifedipine. For IC_{50} values see Table 4.

Table 4 IC_{50} values for prazosin, HV723, WB4101 and benoxathian in inhibiting the adrenergic contractions induced by electrical stimulation in the epididymal portion of rat vas deferens

	IC_{50} (nM)		
	− *Nifedipine*		+ *Nifedipine*
Antagonist	*Phasic*	*Tonic*	*Phasic*
Prazosin	25.7 ± 5.1	25.9 ± 6.0	22.4 ± 3.3
HV723	15.9 ± 2.2	14.9 ± 1.9	15.2 ± 1.6
WB4101	7.27 ± 0.54	7.58 ± 1.39	10.2 ± 0.6
Benoxathian	10.9 ± 1.6	8.66 ± 0.55	9.26 ± 0.76

Electrical transmural stimulation (5 Hz, for 5 s) was applied in the absence or presence of 1 μM nifedipine, and the phasic and tonic components of contraction evoked were measured.
Mean ± s.e. of 4 to 5 experiments.

tions of [^{3}H]-prazosin. In the present study, we used 1 or 10 μM prazosin in the saturation experiments.

In the displacement experiments, the prazosin-high affinity sites were characterized as WB4101- or benoxathian-high affinity sites, while the prazosin-low affinity sites showed a low affinity for WB4101 or benoxathian. HV723, an α_{1N}-selective drug (Oshita *et al.*, 1988; Muramatsu *et al.*, 1990a), did not discriminate between the sites, resulting in low affinity constant.

As mentioned in the Introduction, the α_1-adrenoceptor was originally subdivided into two classes (α_{1A} and α_{1B}) in the binding studies (Morrow & Creese, 1986; Han *et al.*, 1987a) and into three subtypes (α_{1H}, α_{1L} and α_{1N}) in functional studies (Muramatsu *et al.*, 1990a,b). Subsequently, a possible conciliation has been proposed (Oshita *et al.*, 1991; Muramatsu *et al.*, 1991). According to the criteria proposed (Table 1), the characteristics of α_1-adrenoceptor of rat vas deferens observed in the present study show that the prazosin-high and -low affinity sites correspond to the putative α_{1A} and α_{1L} subtypes, respectively. Recently, the existence of mRNA for α_{1A} in the rat vas deferens was confirmed by Northern blotting analysis (Lomasney *et al.*, 1991b).

The functional study reveals that prazosin, HV723, WB4101 and benoxathian competitively antagonize the contractile response to noradrenaline with relatively low affinities ranging from 8.0 to 9.0. Such low affinities for the antagonists have been reported previously (prazosin: Kenakin, 1984; Beckeringh & Brodde, 1990; Salles & Badia, 1991; benoxathian: Han *et al.*, 1987a). However, a higher (approximately 9.5: Han *et al.*, 1987a) or intermediate value (9.01: Hanft & Gross, 1989) was reported for WB4101. At present, we cannot account for this discrepancy because low affinity constants for WB4101 and prazosin were still obtained even after a longer equilibration (60 min) with the antagonist. All of these studies, however, clearly show that the contractile response to noradrenaline of the rat vas deferens is predominantly mediated through a single α_1-adrenoceptor subtype, even though two distinct subtypes co-exist in the vas deferens. Good correlation between the pK_B values for antagonists in the present functional study and the $pK_{I\text{low}}$ values in the binding study strongly suggests that the contractile response to noradrenaline in the rat vas deferens is predominantly mediated through the α_{1L} subtype, although a minor contribution of the α_{1A} subtype cannot be ruled out completely.

It is well known that the epididymal portion produces larger contractions in response to various α-adrenoceptor agonists than the prostatic portion (present study; Vardolv & Pennefather, 1976; Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982; Badia & Salles, 1989). The existence of spare receptors has been demonstrated in the response to noradrenaline in the epididymal but not prostatic portion (Salles & Badia, 1991). The heterogeneous distribution of the α_{1L} but not the α_{1A} subtype observed in the present study may well account for such differences in functional responsiveness between the two portions of rat vas deferens.

CEC was originally reported to produce a selective and complete inactivation of α_{1B} subtype (Han *et al.*, 1987b). However, a partial inactivation of other subtypes by CEC was recently demonstrated (Schwinn *et al.*, 1990; Oshita *et al.*, 1991; Lomansney *et al.*, 1991b). In the present study also, [^{3}H]-prazosin binding to prazosin-high and -low affinity sites (α_{1A} and α_{1L} subtypes) was slightly but significantly inhibited by CEC. The lack of potent inhibitory effect by 10 μM CEC on the contractile responses to exogenous noradrenaline and to adrenergic nerve stimulation (in the presence of the prejunctional α_2-adrenoceptor antagonist, DG-5128; Muramatsu *et al.*, 1989) may be related to low susceptibility to CEC of the α_{1L} subtype which would be predominantly involved in the adrenergic contractile responses as mentioned above.

The contractile response to exogenous noradrenaline and the tonic contraction evoked by electrical transmural stimulation (in the presence of α,β-methylene ATP) were both potently inhibited by nifedipine, whereas the phasic adrenergic response to nerve stimulation was resistant to nifedipine. Such nifedipine-resistance has been observed in the adrenergic but not purinergic contractions induced by single pulse stimulation (Blakeley *et al.*, 1991; Brown *et al.*, 1983; McGrath & Wilson, 1988). This indicates that endogenous noradrenaline may produce contractions through at least two different effector pathways. Since α_{1A}-adrenoceptors were originally suggested to be selectively coupled to Ca channels (Han *et al.*, 1987a), we compared the inhibitory potencies of α_1-adrenoceptor antagonists on the phasic and tonic contractions. Both responses were equipotently inhibited by the antagonists used in the binding study, and the order of inhibitory potencies was consistent with that for inhibition of exogenous noradrenaline-responses or the order of affinity for the α_{1L}-subtype estimated from the binding study. These results indicate that α_1-adrenoceptors involved in both phasic and tonic contractions cannot be discriminated by the competitive antagonists, suggesting that both the response may be caused through the same subtype as that in the response to exogenous noradrenaline (presumably α_{1L}) even though the effector pathways may differ. It seems that α_1-adrenoceptor subtypes cannot be classified strictly by difference in signal transduction mechanisms (Muramatsu *et al.*, 1990b; Lomasney *et al.*, 1991a).

In conclusion, the present study clearly indicates the occurrence of two distinct α_1-adrenoceptor subtypes in the rat vas deferens (presumably α_{1A} and α_{1L}), and suggests that the α_{1L} subtype is predominantly involved in the adrenergic contractions induced by exogenous and endogenous noradrenaline. Variation in adrenergic responsiveness between the epididymal and prostatic portions seems to be related to the heterogeneous distribution of α_{1L} subtypes.

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